1   TITLE OF THE INVENTION
2   Immunogenic Recombinant Antibody
3   CROSS-REFERENCE TO RELATED APPLICATIONS
4   This application is the National Stage Application of Interna-
5   tional Patent Application No. PCT/EP2004/004059 filed on April
6   16, 2004, which claims priority on application No. A 599/2003
7   filed in Austria on April 17, 2003, the entire contents of which
8   are hereby incoprorated by reference.
9
10
11   BRIEF SUMMARY OF THE INVENTION
12   The invention refers to an immunogenic recombinant antibody that
13   is used for immunization of primates, in particular human be-
14   ings. The invention further refers to a vaccine comprising the
15   immunogenic recombinant antibody, and a method of producing the
16   same.
17
18
19   BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)
20   Figure 1: Figure of the original pIRES expression vector
21   Figure 2: Figure of the cloning cassette of the tri-cistronic
22   mAb17-1A expression and DHFR selection construct.
23   Figure 3: Sequence of the cloning cassette of the tri-cistronic
24   mAb 17-1A expression and DHFR selection construct, introduced
25   restriction sites bold and italic; KOZAK sequences underlined.
26   Figure 4: Figure of an IgG2a Le-Y antibody
27   Figure 5: Molecular biological IgG2a Le-y antibody construct
28   Figure 6: amino acid sequence of mAb17-1A gamma
29   Figure 7: Amino acid sequence of mAb17-1A kappa
30   Figure 8: Amino acid sequence of mAb17-1A kappa with Arginine
31   instead of Lysine at position 146
32   Figure 9: Amino acid sequence of mAb17-1A kappa with Arginine
33   replacements outside the CDRs
34   Figure 10: Cross-comparative ELISA analysis. Geometric means (4
35   animals per group) and CI (95%) are shown.
36
37   DETAILED DESCRIPTION OF THE INVENTION
38
39   The invention refers to an immunogenic recombinant antibody that
40   is used for immunization of primates, in particular human be-

1 ings. The invention further refers to a vaccine comprising the

2 immunogenic recombinant antibody, and a method of producing the

3 same.

4

5 Monoclonal antibodies (MAB) have been widely used for immuno-

6 therapy of a variety of diseases, among them infectious and

7 autoimmune disease, as well as conditions associated with tu-

8 mours or cancer. Using hybridoma technology MAB directed against

9 a series of antigens have been produced in a standardized man-

10 ner. A multitude of tumor-associated antigens (TAAs) are consid-

11 ered suitable targets for MAB and their use for the diagnosis of

12 cancer and therapeutic applications. TAAs are structures that

13 are predominantly expressed on the cell membrane of tumor cells

14 and thus allow differentiation from non-malignant tissue.

15

16 Whether human TAAs detected by xenogeneic MABs are capable of

17 inducing an antitumor immune response in cancer patients, and

18 whether such antigens are indeed related to the response to

19 autologous tumors in cancer patients, depends on the nature of

20 the respective TAA and is still not fully understood. TAAs which

21 are either naturally immunogenic in the syngeneic host or can be

22 made immunogenic might potentially be used to induce antitumor

23 immunity for therapeutic and possibly prophylactic benefit.

24

25

26 For passive immunotherapy MABs are administered systemically to

27 a patient in a suitable amount to directly bind to a target.

28 Thus an immune complex is formed and through a series of immune

29 reactions the cell or organism afflicted with the target is

30 killed. The therapeutic effect is depending on the concentration

31 of the MABs in the circulation and the biological half-life,

32 which is usually quite short. It is therefore necessary to re-

33 peat the administration within an appropriate timeframe. If

34 xenogeneic MABs, such as murine antibodies are used, adverse re-

35 actions are however expected, possible leading to anaphylactic

36 shock. Therefore, such immunotherapies are employed for a lim-

37 ited time only.

38

39 Active immunization regimens activate the immune system of pa-

40 tients in a specific way. Following the administration of an

1   antigen that resembles a specific target the patients humoral

2   and T-cell specific immune response induces defense mechanisms

3   to combat the target in vivo. For active immunization these an-

4   tigens are usually presented in an immunogenic formulation to

5   provide a vaccine. Antigens mimicking the targets have either

6   similarities in the primary and secondary sequence of the tar-

7   gets or fragments thereof. Mimotopes or mimotopic antigens, how-

8   ever, have similarities in the tertiary structure of the target.

9

10   Exemplary mimotopes are anti-idiotypic antibodies or mimotopic

11   antibodies that imitate the structure of an antigen, which is

12   considered as target for the immune system. Idiotypic interac-

13   tions strongly influence the immune system. The unique antigenic

14   determinants in and around the antigen-combining site of an im-

15   munoglobulin (Ig) molecule, which make one antibody distinct

16   from another, are defined as idiotopes. All idiotopes present on

17   the variable portion of an antibody are referred to as its idio-

18   type (id). The molecular structure of an idiotype has been lo-

19   calized to both the complementary determining regions and the

20   framework regions of the variable domain and is generally but

21   not always contributed to by both the heavy and the light chains

22   of an immunoglobulin in specific association.

23

24   Idiotypes are serologically defined entities. Injection of an

25   antibody (Ab1) into a syngeneic, allogeneic, or xenogeneic re-

26   cipient induces the production of anti-idiotypic antibodies

27   (Ab2). With regard to idiotype/anti-idiotype interactions a re-

28   ceptor-based regulation of the immune system was postulated by

29   Niels Jerne (Ann. Immunol. 125C, 373, 1974). His network theory

30   considers the immune system as a collection of lg molecules and

31   receptors on T-lymphocytes, each capable of recognizing an anti-

32   genic determinant (epitope) through its combining site (para-

33   tope), and each capable of being recognized by other antibodies

34   or cell-surface receptors of the system through the idiotopes

35   that it displays.

36

37   Many studies have indeed demonstrated that idiotypic and anti-

38   idiotypic receptors are present on the surface of both B- and T-

39   lymphocytes as well as on secreted antibodies. An overview about

40   anti-idiotypic antibodies used for the development of cancer

1   vaccines is presented by Herlyn et al. (in vivo 5: 615-624

2   (1991)). The anti-idiotypic cancer vaccines contain either mono-

3   clonal or polyclonal Ab2 to induce anti-tumor immunity with a

4   specificity of selected TAA.

5

6   When the binding between Ab1 and Ab2 is inhibited by the antigen

7   to which Ab1 is

8   directed, the idiotype is considered to be binding-site-related,

9   since it involves a site on the antibody variable domain that is

10 engaged in antigen recognition. Those idiotypes which conforma-

11 tionally mimic an antigenic epitope are called the internal im-

12 age of that epitope. Since both an Ab2 and an antigen bind to

13 the relevant Ab1, they may share a similar three-dimensional

14 conformation that represents the internal image of the respec-

15 tive antigen. Internal image anti-idiotypic antibodies in prin-

16 ciple are substitutes for the antigen from which they have been

17 derived via the idiotypic network. Therefore these surrogate an-

18 tigens may be used in active immunization protocols. The anti-

19 idiotypic antibodies offer advantages if the original antigen is

20 not sufficiently immunogenic to induce a significant immune re-

21 sponse. Appropriate internal image anti-idiotypic antibodies

22 that mimic a non-immunogenic carbohydrate antigen are especially

23 useful for certain vaccination approaches.

24

25 Tumor associated antigens are often a part of "self" and evoke a

26 very poor immune response in cancer patients. In contrast, in-

27 ternal image anti-idiotypic antibodies expressing three-

28 dimensional shapes, which resemble structural epitopes of the

29 respective TAA, are recognized as foreign molecules in the tu-

30 mor-bearing host.

31

32 The immune response raised by therapeutic or even prophylactic

33 immunization with appropriate anti-id MABs, thus may cause anti-

34 tumor immunity.

35

36 Mimotopic antibodies are alike anti-idiotypic antibodies. They

37 too resemble a target structure and may possibly activate the

38 immune system against the target. The EP-B1-1 140 168 describes

39 mimotopic antibodies against human cellular membrane antigens to

40 produce antitumor immunity in cancer patients. These antibodies

are directed against the EpCAM, NCAM or CEA antigens; each of these targets is well known to be tumor associated.

Therapeutic immunization against cancer with MABs may be especially successful in earlier stages of the disease: At the time of surgery of a primary tumor, frequently occult single tumor cells already have disseminated in various organs of the patient. These micrometastatic cells are known to be the cause for the later growth of metastases, often years after diagnosis and surgical removal of all clinically proven tumor tissue. So far in almost all cases metastatic cancer of epithelial origin is incurable.

Therefore an effective treatment of "minimal residual cancer", e.g. destruction of occult disseminated tumor cells or micrometastatic cells in order to prevent the growth of metastases is an urgent medical need. At these stages of the disease (adjuvant setting) conventional chemotherapeutic approaches are rather unsuccessful. However, specific antitumor immunity at the time of minimal residual disease can be obtained by immunization with appropriate MAB. Micrometastatic cells may thus be selectively eliminated by the immune system, leading to an increased relapse-free survival time.

Monoclonal antibodies with the specificity of BR55-2 (disclosed in e.g. Wistar EP 285 059, M.Blaszcyk-Thurin et al., J.Biol.Chem. 262 (1987) 372-379, or Z.Steplewski et al., Hybridoma 9 (1990) 201-210) bind to the Lewis Y6 antigen, a carbohydrate determinant selectively expressed on a majority of human solid tumors. Based on their properties antibodies BR55-2 can be used for passive immunotherapy of epithelial cancer.

The tumor associated Lewis Y oligosaccharide determinant, which is also expressed during certain stages of embryonic development, is almost not immunogenic by itself. However, monoclonal anti-idiotypic antibodies (Ab2) against BR55-2 (Ab1) with internal image properties by resembling structural epitopes of the Lewis Y antigen are useful for induction of a protective antitumor immunity, particularly in earlier stages of the disease (EP-B1-0 644 947).

1

2 Monoclonal anti-idiotypic antibodies (Ab2) against BR55-2 (Ab1)

3 with internal image properties are described in EP-B1-0 644 947

4 to be used for inducing immunity against both free HIV and HlV-

5 infected cells.

6

7 In addition to its expression on cancer of epithelial origin the

8 Lewis Y carbohydrate antigen is also involved in the pathogene-

9 sis of infection with Human Immunodeficiency Virus (HIV). HIV-

10 infected cells in vitro and in vivo express on their surface an

11 altered glycosylation pattern, namely the Lewis Y carbohydrate

12 determinant. This antigen normally occurs only during certain

13 fetal development stages and is also associated with a variety

14 of malignancies. Expression on HlV-infected cells may reflect

15 their altered differentiation status induced by retroviral

16 transformation. The Lewis Y oligosaccharide represents a spe-

17 cific host response expressed both on HlV-infected cells and

18 free HlV-particles.

19

20 EpCAM (Epithelial Cell Adhesion Molecule) is expressed on nearly

21 all tumors of epithelial origin, but also occurs on a large num-

22 ber of normal epithelial tissue or epithelial cells. It has been

23 characterized as a self-adhesion molecule and is classified as a

24 pan-epithelial adhesion antigen (J. Cell Biol. 125: 437 (1994)).

25 As a membrane-anchored glycoprotein it strongly interacts in

26 cell-to-cell adhesion in cancerous tissues.

27

28 Human epithelial antigen EpCAM derived peptides are proposed for

29 treatment or prophylaxis of EpCAM associated cancers, for induc-

30 tion of cytotoxic T lymphocyte response effective against EpCAM

31 positive tumor cells and for diagnostic purposes (WO-A1-

32 97/15597).

33

34 US-B1-6 444 207 describes an immunotherapy of tumors with a hy-

35 bridoma derived monoclonal antibody against the 17-1A antigen,

36 which is a determinant of the EpCAM molecule. Multiple doses of

37 about 400 mg or more are administered for passive immunotherapy

38 of gastrointestinal cancer.

39

40 EP-B1-1 140 168 describes an immunogenic formulation of HE2, an

1   EpCAM specific murine IgG2a antibody. Immunization studies
2   proved the induction of a strong antigen specific immune re-
3   sponse cross-reacting with EpCAM and activating complement fac-
4   tors to induce tumor cell lysis. Rhesus monkey studies and
5   clinical data indicated a high immunogenicity of the HE2 immuni-
6   zation antigen.
7
8   The expression of recombinant proteins in higher eukaryotic
9   cells represents an essential tool in modern biology. The re-
10   finement of mammalian gene expression vectors enabled the pro-
11   gress in diverse scientific fields (Makrides, Protein Expression
12   and Purification 17: 183-202 (1999)). Due to the increased de-
13   mand for human antibodies to be used for human therapy, studies
14   concerning the suitable cell line for high yield production of
15   such complex molecules have been performed. Human or human-mouse
16   hetero-hybridomas often have some limitations such as low growth
17   rates and high serum requirements. This has led to the alterna-
18   tive use of recombinant cells to produce recombinant antibodies
19   with the advantages of selection of cell lines for transfection,
20   control of the antibody isotype, control of expression using
21   strong promoters, etc (Strutzenberger et al., J Biotechnology
22   69(2-3): 215-26 (1999)). The standard model of protein transla-
23   tion applies to the vast majority of eukaryotic mRNAs and in-
24   volves ribosome entry at the 5´cap structure followed by scan-
25   ning of the mRNA in 5´to 3´direction until the initiation codon
26   is reached. In the field of IgG expression, the biomolecule is
27   assembled by 4 correctly folded subunits. Amount and localiza-
28   tion of these different subunits strongly influences folding by
29   self-organization of the expression product and therefore its
30   biological activity.
31
32   US-B1-6 331 415 describes methods of producing recombinant immu-
33   noglobulins, vectors and transformed host cells. One or more
34   vectors are used to produce both heavy and light chains of an
35   antibody, or fragments thereof in a single cell. Disclosed hosts
36   are bacterial cells or yeast.
37
38   Due to different amounts of the genes encoding the immunoglobu-
39   lin subunits integrated into the host genome, misfolded and bio-
40   logical inactive expression products may occur. It is required

1 that two different genes are transcribed and four polypeptide
2 chains are assembled in a balanced manner. Therefore oligocis-
3 tronic expression systems are described for the production of
4 antibodies (WO-A1-98/11241). The oligocistronic expression vec-
5 tors are under the control of a strong promoter/enhancer unit, a
6 selection marker gene and at least two IRES (Internal Ribosomal
7 Entry Site) elements.
8
9 Bi-cistronic expression vectors may be suitable for a balanced
10 expression of the polypeptide chains. IRES elements are usually
11 derived from encephalomyocarditis virus, foot-and-mouth disease
12 virus or poliovirus. Ribosomes are able to enter a mRNA molecule
13 at the IRES sites and initiate the translation of multiple open
14 reading frames on the same mRNA strand. The major advantage of
15 those constructs is the possibility to express different genes
16 under the control of a single promoter independent from their
17 integration sites into the host genome. Selection markers inte-
18 grate independent of the desired genes to be expressed into the
19 host genome (Rees S. et al., BioTechniques, 1996, 20, 103-110).
20
21 In order to overcome possible problems of repeated use of murine
22 antibodies for treating humans, mouse/human chimeric MABs can be
23 generated by combining the variable domains of a parent murine
24 MAB of choice with human constant regions. To further improve
25 the properties of MABs for use in passive immunotherapy, "fully
26 humanized" antibodies are constructed by recombinant DNA tech-
27 nology. Minimal parts of a parent mouse antibody that comprise
28 the complementarity determining regions (CDRs), are combined
29 with human variable region frameworks and human constant re-
30 gions. For the design and construction of these "fully human-
31 ized" MABs, sequence homology and molecular modelling is used to
32 select a combination of mouse and human sequence elements that
33 would further reduce immunogenicity while retaining the binding
34 properties.
35
36 Schneider et al (Proc Natl Acad Sci USA 85: 2509-13 (1988)) de-
37 scribe genetically engineered immunoglobulins revealing struc-
38 tural features that control segmental flexibility of an immu-
39 noglobulin. The proteins studied were hybrids of relatively
40 rigid isotype (mouse IgG1) and a relatively flexible one (mouse

1 IgG2a).

2

3 It was the object of the invention to provide preparations of

4 monoclonal antibodies with improved immunogenic properties to be

5 used for immunizing patients, in particular cancer patients.

6

7 According to the invention there is provided an immunogenic re-

8 combinant antibody that is designed for immunization of pri-

9 mates. The antibody comprises at least part of a murine IgG2a

10 subtype amino acid sequence and a mammalian glycosylation. The

11 antibody according to the invention is obtained by recombinant

12 nucleic acid technology, in particular recombinant DNA technol-

13 ogy, to produce the immunogenic antibody in a standardized man-

14 ner.

15

16 Immunization studies surprisingly revealed that the murine IgG2a

17 part is critical to design an immunogenic antibody, in particu-

18 lar when compared to IgG1 antibodies. In the following the immu-

19 nogenic antibody comprising at least part of the IgG2a amino

20 acid sequence according to the invention is called "IgG2a immu-

21 nogenic antibody".

22

23 The term "immunogenic" defines any structure that leads to an

24 immune response in a specific host system. For example, a murine

25 antibody or fragments thereof is highly immunogenic in humans,

26 especially when combined with adjuvants.

27

28 An immunogenic antibody according to the invention may have im-

29 munogenicity by its specificity or by its structure. The immuno-

30 genic antibody can induce immunogenicity also when being dena-

31 tured or when conjugated to certain structures or carriers.

32

33 The humoral immune response induced by the IgG2a immunogenic an-

34 tibodies according to the invention has significantly improved

35 in terms of the quantity of specific antibody induced by the pa-

36 tients and the specificity against selected targets and epi-

37 topes. The improved immune response surprisingly turned out to

38 be dependent on the glycosylation pattern of the antibody. A

39 non-glycosylated or deglycosylated variant of the IgG2a immuno-

40 genic antibody according to the invention can also induce an im-

1 mune response, although the immune response is lower and/or the
2 immunization kinetics is delayed compared to a glycosylated an-
3 tibody. A similar titer endpoint can be deserved but individuals
4 take significantly longer to reach plateau values of immuniza-
5 tion antigen specific titers.
6
7 It was surprisingly found by the inventors that a recombinant
8 antibody expressed in hamster or human cells shows a similar im-
9 munogenicity than an antibody expressed by murine hybridoma
10 cells. This is of particular relevance for antibodies that are
11 used for immunization purposes.
12 It was well known in the art that immunogenicity of antigens is
13 highly influenced by the glycosylation pattern. In case of tumor
14 vaccines a major prerequisite for their success is their uptake
15 by antigen-presenting cells (APCs) and transport of these APCs
16 to the draining lymph nodes where the processed and presented
17 tumor-associated antigens activate tumor-specific naïve T-cells.
18 This immunogenicity is highly increased by α-Gal epitopes (Gal α
19 1,3Galß1,4GlcNAc-R, Galili-epitopes). The α-gal-epitope is pro-
20 duced in large amounts in non-primate mammals and New world mon-
21 keys, but it is completely absent in humans, apes and Old World
22 monkeys, because these species lack □1,3Galactosyltransferase.
23 Also CHO cells do not express these Galili epitopes (La Temple
24 D.C. et al., 1999, Cancer Res.,59, 3417-3423, Winand R.J. et al,
25 J. Immunol., 1993, 151, 3923-3934).
26
27 Nevertheless, CHO (Chinese hamster ovary) or human glycosylation
28 has proven to provide an immunogenic antibody that can be supe-
29 rior to a non-glycosylated variant. Glycosylation patterns of
30 rodents or those of primates, among them human or chimpanzees,
31 are preferred. Preferably the rodents are non-murine.
32
33 The antibody may have a murine amino acid sequence or any other
34 mammalian amino acid sequence that is combined with the murine
35 IgG2a part. Preferable mammalian sequences are human or human-
36 ized or human/murine chimeric or murine sequences. Among the
37 preferred antibodies are thus murine, chimeric or humanized and
38 "fully humanized" antibodies.
39
40 The IgG2a immunogenic recombinant antibody according to the in-

1 vention can be an antibody directed against a tumor associated
2 antigen (TAA) or a part or fragment thereof.
3
4 The IgG2a immunogenic antibody according to the invention can
5 also be an anti-idiotypic antibody (Ab2) or a mimotopic Ab1 an-
6 tibody. Either the functional antibody is provided, or frag-
7 ments, variants and derivatives thereof. A functional antibody
8 consists of two types of polypeptide chains that can be cleaved
9 into further subunits, the two large, heavy chains and two light
10 chains. The polypeptides are connected by disulfide bridges and
11 non-covalent bounds. The light chains are either lambda or kappa
12 chains. Preferably the functional antibody has a natural speci-
13 ficity and can activate the complement system. More preferably
14 it has neutralizing activity.
15 The mimotopic antibody according to the invention preferably
16 mimics an antigen or target that is recognized by the idiotype
17 of the antibody itself. The idiotypic antibody (Ab1) is prefera-
18 bly directed against a tumor-associated antigen, TAA. The pre-
19 ferred Ab2 antibody according to the invention is directed
20 against the idiotype of an antibody specific for a TAA.
21
22 The IgG2a immunogenic antibody according to the invention may
23 present the specific epitopes, which are either present in the
24 mammalian original amino acid sequence or introduced by antibody
25 engineering, including recombination, conjugation and derivati-
26 zation techniques.
27
28 Generally, a molecular modelling to redesign the antibody ac-
29 cording to the invention can be carried out. The possible varia-
30 tions are many and range from the changing of just one or a few
31 amino acids to the complete redesign of, for example, the con-
32 stant region. Changes in the constant region will, in general,
33 be made in order to improve the cellular process characteris-
34 tics, such as complement fixation, interaction with membranes,
35 and other effector functions. Changes in the variable region
36 will be made in order to improve the antigen binding character-
37 istics. These alterations can be made by standard recombinant
38 techniques and also by oligo-directed mutagenesis techniques
39 (Dalbadie-McFarland et al., Proc.Natl.Acad.Sci (USA), 79:6.409
40 (1982), WO 91/17177, Berstein et al., J.Mol.Biol., 112:535-542

1   (1977)

2

3   The amino acid sequence of the IgG2a antibody according to the

4   invention can be identical to the mammalian original amino acid

5   sequence but can also include amino acid variations leading to

6   an IgG2a antibody with immunogenic properties comparable, pref-

7   erably identical to those of the IgG2a antibody containing the

8   mammalian original amino acid sequence.

9   For example, the amino acid variations can be a variation of one

10   or more amino acids, preferably not more than ten amino acids,

11   more preferably not more than 5 amino acids, most preferably one

12   amino acid compared to the sequence of an IgG2a antibody as

13   known from Sun et al. (Proc Natl Acad Sci USA, 84:214-8 (1987))

14   or according to Figure 6 or 7.

15

16   The amino acid of the kappa chain can be as shown in Fig. 8.

17   Alternatively there is an amino acid variation within the kappa

18   chain of the antibody, preferably approx. 10 amino acids after

19   the end of the 3rd complementarity determining region (CDR). The

20   amino acid variation can be any amino acid, preferably the re-

21   placement of a lysine by an arginine.

22

23   Alternatively there can be replacements of additional and/or

24   other lysine-residues within the kappa chain of the antibody by

25   arginine, for example at positions 9, 38, 53, 68, 74, 132 of

26   Fig. 9.

27

28   These amino acid replacements can lead to the positive effect

29   that the variable region of the antibody contains less primary

30   amines which are preferentially used for covalent protein immo-

31   bilization or coupling of functional groups like carbohydrates

32   via primary amines.

33

34   The term "epitope" defines any region of a molecule that can be

35   recognised by specific antibody or that provoke the formation of

36   those specific antibodies. Epitopes may be either conforma-

37   tional epitopes or linear epitopes.

38

39   Preferred epitopes presented by the IgG2a immunogenic antibody

40   are derived from antigens specific for epithelial tumors (tumor

associated antigens), and frequently expressed in breast cancer, gastrointestinal, colorectal, prostate, pancreatic, and ovary and lung cancer, either being small cell lung cancer (SCLC) or non small cell lung cancer (NSCLC). The preferred epitopes espe-cially induce humoral immune response and the formation of spe-cific antibodies in vivo. The antibodies according to the inven-tion preferably also induce T cell specific response. This can preferably be induced by coupling carbohydrate residues on the antibody according to the invention, such as Lewis antigens, e.g. Lewis x-, Lewis b- und Lewis y-structures, also sialylated Lewis x-structures, GloboH-structures, KH1, Tn-antigen, TF-antigen and alpha-1-3-galactosyl-epitope.

Among the preferred epitopes are protein epitopes that are ex-pressed on malignant cells of solid tumors, e.g. TAG-72, MUC1, Folate Binding Protein A-33, CA125, HER-2/neu, EGF-receptors, PSA, MART etc. Moreover, T cell epitope peptides or mimotopes of such T cell epitopes may be presented by the antibody according to the invention. Suitable epitopes are usually expressed in at least 20% of the cases of a particular disease or cancer, pref-erably in at least 30%, more preferably in at least 40%, most preferably in at least 50% of the cases.

According to the invention there are preferred carbohydrate epi-topes that are derived from tumor associated aberrant carbohy-drate structures, such as Lewis antigens, e.g.
Lewis x-, Lewis b- und Lewis y-structures, also sialylated Lewis x-structures, GloboH-structures, KH1, Tn-antigen, Sialyl-Tn, TF-antigen and alpha-1-3-galactosyl-epitope.

The preferred TAA targets or epitopes are selected from the group of determinants derived from the group of antigens con-sisting of peptides or proteins, such as EpCAM, NCAM, CEA and T cell peptides, carbohydrates, such as aberrant glycosylation patterns, Lewis Y, Sialyl-Tn, Globo H, or glycolipids, such as GD2, GD3 und GM2. Antibodies according to the invention can have or mimic an epitope of any such TAA, and, at the same time, are directed against another or the same TAA, for example a mimo-topic antibody directed against a cellular adhesion molecule, such as EpCAM, NCAM or CEA. These antibodies can be defined as

1   bi-epitopic antibodies or bi-epitopic immunization antigens.

2

3   Additionally the antibody according to the invention can contain

4   a mimotope or mimotopic antigen(s) or antigenic structure(s)

5   triggering immune response specific for tumor associated anti-

6   gens, for example epithelial cell specific adhesion molecules or

7   tumor associated carbohydrate structures. For example,, the

8   IgG2a antibody according to the invention induces the develop-

9   ment of Ep-CAM specific antibodies. Preferably, the antibody ac-

10   cording to the invention can contain an EpCAM specific hinge re-

11   gion.

12

13   It was found that the amino acid sequence of the IgG2a hinge re-

14   gion has structures of homology compared to the Ep-CAM amino

15   acid sequence. The amino acid sequence numbering used is identi-

16   cal to the numbering as published by Strnad J. et al., Cancer

17   Res., 49 (1989), 314-317. These homologies might influence the

18   specificity of the antibody according to the invention for Ep-

19   CAM. For example, amino acids 36 to 42, amino acids 117 to 131,

20   amino acids 124 to 134, amino acids 144 to 160 show significant

21   homology between 29% and 57% to regions within the hinge region

22   of IgG2a antibodies.

23

24   Further preferred antigens or targets are derived from antigens

25   of infectious agents such as viral, bacterial, fungal, transmis-

26   sible spongiform encephalitis agents (TSE) or parasitic agents.

27   Among the preferred antigens or targets are determinants of gly-

28   cosylation patterns of the virus and infected cells, such as

29   Lewis Y glycosylation of

30   infected HIV cells.

31

32   There are methods known in the art to define suitable antigens,

33   determinants and related epitopes necessary to produce the pep-

34   tides, polypeptides or proteins, related nucleic acids, lipopro-

35   teins, glycolipids, carbohydrates or lipids, which are derived

36   from TAA or infectious agents. Without undue experiments the

37   IgG2a immunogenic antibody is thus designed and engineered by

38   selecting the suitable Ab1 mimotopic or Ab2 antibody, optionally

39   modifying its amino acid sequence, and expressing it in a suit-

40   able recombinant host cell.

The IgG2a immunogenic antibody according to the invention may be
specifically designed to have characteristics of composite or
hybrid antibodies to combine at least two types or subtypes of
immunoglobulins. The preferred bi-isotypic antibody is for in-
stance selected from variable regions of IgG1 or IgG3 antibodies
that care switched to the IgG2a subtype amino acid sequence. The
IgG2a subtype amino acid sequence is either inserted into the
sequence of the parent antibody or substitutes for similar parts
of the parent antibody. The preferred location of the IgG2a se-
quence is in the constant region of the antibody, most preferred
in at least one of the regions selected from the group consist-
ing of the CL, CH1, hinge, CH2 and CH3 regions. Most preferred
is an antibody wherein the IgG2a region is within the hinge re-
gion.

The best mode of the IgG2a immunogenic antibody refers to an
anti-idiotypic antibody to monoclonal antibodies produced by
ATCC HB 9324 or ATCC HB 9347, hybridised with at least part of a
murine amino acid sequence of an IgG2a antibody. The IgG2a immu-
nogenic antibody is for example a construct of an anti-idiotypic
Lewis-Y mimicking hypervariable region and the highly immuno-
genic mouse IgG2a constant regions to build a functional anti-
body.

The invention further encompasses vaccines for immunization pur-
poses, which comprise the IgG2a immunogenic antibody in a phar-
maceutical formulation. The pharmaceutical formulation prefera-
bly contains auxiliary agents or adjuvants to improve the qual-
ity of an injection preparation in terms of safety, tolerability
and immunogenicity. The design of the vaccine depends on the
primates that are treated, among them specifically human beings
or chimpanzees.

The vaccines according to the invention may be suitably used for
the prophylaxis and therapy of cancer associated diseases, e.g.
metastatic disease in cancer patients. The vaccine according to
the invention specifically modulates antigen presenting cells in
vivo or ex vivo, thus generating immune response to the epitope
that is targeted by the IgG2a immunogenic antibody.

1

A vaccine according to the invention typically contains the
IgG2a immunogenic antibody at low concentrations. The immuno-
genic amount often is ranging between 0.01 μg and 10 mg/single
dose. Depending on the nature of the antibody, the immunogenic-
ity may be altered by xenogenic sequences or derivatization of
the antibody. Besides, the use of adjuvants further increases
the immunogenicity of the IgG2a antibody. The immunogenic dose
of an antibody suitably formulated with an adjuvant is thus
preferably ranging between 0.01 μg and 750 μg/single dose, most
preferably between 100 μg and 500 μg/single dose. A vaccine de-
signed for depot injection will however contain far higher
amounts of the IgG2a immunogenic antibody, e.g. at least 1 mg up
to 10 mg/single dose. The immunogen is thus delivered to stimu-
late the immune system over a longer period of time.

The vaccine according to the invention usually is provided as
ready-to-use preparation in a single-use syringe containing a
volume of 0.01 to 1 ml, preferably 0.1 to 0.75 ml. The vaccine
solution or suspension thus provided is highly concentrated. The
invention further relates to a kit for vaccinating patients,
which comprises the vaccine and suitable application devices,
such as a syringe, injection devices, pistols. etc.

The vaccine is specifically formulated to produce a pharmaceuti-
cal preparation suitable for subcutaneous, intramuscular, in-
tradermal or transdermal administration. Another possible route
is the mucosal administration, either by nasal or peroral vacci-
nation. If solids are used to prepare the pharmaceutical formu-
lation the IgG2a immunogenic antibody is either administered as
adsorbate or in suspension with the solids. Particular embodi-
ments contain aqueous media for suspending the formulation or
for solutions of the IgG2a immunogenic antibody to provide a
liquid vaccine.

The vaccine is usually storage stable at refrigerating tempera-
ture. However, preservatives, such as thimerosal or other agents
of improved tolerability may be used to improve its storage sta-
bility to enable prolonged storage times even at elevated tem-
peratures up to room temperature. The vaccine according to the

1 invention may also be provided in the frozen or lyophilized
2 form, which is thawed or reconstituted on demand.
3
4 Preferred pharmaceutical formulations contain pharmaceutically
5 acceptable carrier, such as buffer, salts, proteins or preserva-
6 tives.
7
8 Exemplary adjuvants improving the efficacy of the vaccine ac-
9 cording to the invention are aluminium hydroxide (alum gel) or
10 aluminium phosphate, such as growth factors, lymphokine, cyto-
11 kines, like IL-2, IL-12, GM-CSF, gamma interferon, or complement
12 factors, e.g. C3d, liposomal preparations and formulations of
13 additional antigens that are strong immunogens, such as tetanus
14 toxoid, bacterial toxins, like pseudomonas exotoxins, Bacillus
15 calmette Guerin (BCG) and derivatives of Lipid A.
16
17 In addition methods for producing antibody conjugates or dena-
18 tured vaccine components may be employed to increase the immuno-
19 genicity of the IgG2a immunogenic antibody. Mixtures of the
20 IgG2a immunogenic antibody and further vaccine antigens, in par-
21 ticular different anti-idiotypic antibodies, may serve for si-
22 multaneous vaccination.
23 The IgG2a immunogenic antibody is produced by genetic engineer-
24 ing as a recombinant molecule. Suitable host cells are CHO (Chi-
25 nese hamster ovary) cells, BHK (baby hamster kidney) cells, HEK
26 (human embryonic kidney) cells or the like. In any case the
27 translated antibody thus obtains the glycosilation pattern of
28 the host cell, which is critical to the immunogenicity of the
29 antibody. If a host cell is selected that produces no glycosyla-
30 tion (such as bacterial cells, like E. coli) the antibody may be
31 glycosylated by chemical or enzymatic means. The glycosylation
32 pattern may be altered by common techniques.
33
34 Specific host cells may be selected according to their capabil-
35 ity to produce a glycosylated expression product. Host cells
36 could also be modified to produce those enzymes that are re-
37 quired for a specific glycosylation (Glycoconj. J. (1999), 16:
38 81).
39
40 Host cells expressing the antibody according to the invention

1 are preferably cultivated without using serum or serum compo-
2 nents. Common cultivation media may contain bovine serum, thus
3 introducing bovine immunoglobulins into the harvested medium.
4 Those bovine immunoglobulins or IgG may be difficult to separate
5 from the expression product, which is the IgG2a immunogenic an-
6 tibody according to the invention. Thus, the expression product
7 is preferably obtained by cultivating host cells in a serum free
8 medium, i.e. without the use of bovine serum, to produce an an-
9 tibody devoid of bovine IgG, as measured by HPLC methods.
10
11 The IgG2a immunogenic antibody may have a native structure of a
12 functionally intact antibody. However, it might be advantageous
13 to produce an antibody derivative, preferably selected from the
14 group of antibody fragments, conjugates or homologues. Preferred
15 derivatives contain at least parts of the Fab fragment, most
16 preferably together with at least parts of the F(ab´)2 fragment
17 and/or parts of the hinge region and/or parts of the Fc region
18 of a lambda or kappa antibody. These fragments may be produced
19 according to methods known from prior art, e.g. cleaving a mono-
20 clonal antibody with proteolytic enzymes such as papain or pep-
21 sin, or by recombinant methods. These Fab and F(ab)2 fragments
22 may also be prepared by means of phage display gene library
23 (Winter et al., 1994, Ann.Rev.Immunol., 12:433-455).
24 The IgG2a immunogenic antibody according to the invention is
25 usually of an IgG, IgM or IgA type.
26
27 Moreover, a single chain antibody derivative might be used as
28 IgG2a immunogenic antibody according to the invention.
29
30 The preferred method for producing an antibody according to the
31 invention makes use of a multicistronic antibody-expression con-
32 struct to be used in a CHO, BHK or primate expression system.
33 The construct according to the invention contains at least a nu-
34 cleotide sequence encoding a kappa light chain and at least a
35 nucleotide sequence encoding a gamma heavy chain, wherein at
36 least one of the nucleotide sequences encoding a kappa light
37 chain or gamma heavy chain comprises a nucleotide sequence en-
38 coding at least part of a murine IgG2a subtype amino acid se-
39 quence, and at least two IRES elements. Thus, the polypeptide
40 chains of the antibody are expressed in a balanced manner.

1

2 The nucleotide sequence encoding at least the part of the murine
3 IgG2a subtype amino acid sequence is preferably ligated into the
4 nucleotide sequence encoding the kappa light chain or the gamma
5 heavy chain by one of insertion or substitution techniques to
6 obtain an antibody expression construct. The nucleotide sequence
7 encoding the kappa chain and a nucleotide sequence encoding the
8 gamma chain are preferably linked by an IRES sequence.

9

10 A vector according to the invention comprises a promotor, an an-
11 tibody-expression construct as described above and a transcrip-
12 tion termination sequence. The vector preferably contains one of
13 the IRES sequences in the attenuated form. Through an inserted
14 sequence the IRES sequence may be attenuated to downregulate the
15 entry of the ribosomes and the expression of a quantitative se-
16 lection marker operatively linked thereto. Thus, those host
17 cells that produce the selection marker and the expression prod-
18 uct at the highest level can easily be selected. The IRES se-
19 quence is preferably attenuated by insertion of the sequence to
20 locate it pre and/or post the IRES sequence. The insertion se-
21 quence may encode a hairpin.

22

23 Insertion of overhangs/IRES flaniing regions that significantly
24 reduce efficiency of (cap-independent) initiation of translation
25 might be of preference.

26

27 Among the preferable selection markers there is the DHFR (dihy-
28 drofolate reductase) gene, which is an essential component for
29 the growth of transfected DHFR deficient CHO cells in the pres-
30 ence of MTX (methotrexate). Alternatively, also other selection
31 and amplification markers can be used, such as hygromycin-B-
32 phosphotransferase, thymidine kinase etc. Using an IRES sequence
33 a selection marker will integrate exactly at the same site as
34 the foreign gene and selection will occur on the same mRNA en-
35 coding for both antibody chains and also the selection marker.
36 By attenuating this second IRES sequence, translation efficiency
37 of the selection marker will strongly be reduced. The use of a
38 DHFR deficient CHO strain enables selection and gene copy number
39 amplification using low selective concentrations of MTX ranging
40 from 1 to 10 μmol/l.

1

2 A bicistronic pIRES expression vector is commercially available

3 (Clontech laboratories Inc, Palo Alto, USA). This construct can

4 be modified to produce the heavy and light antibody chains at

5 nearly the same high expression levels.

6

7 The preferred method of producing an antibody according to the

8 invention comprises the steps of

9 transforming a CHO host cell with a multicistronic antibody-

10 expression construct containing at least a nucleotide sequence

11 encoding a kappa light chain and a nucleotide sequence encoding

12 a gamma heavy chain, wherein at least one of the nucleotide se-

13 quences comprises a nucleotide sequence encoding at least a part

14 of a murine IgG2a subtype amino acid sequence, and at least two

15 IRES elements, and

16 expressing said nucleotide sequences of immunoglobulines under

17 the control of a single CMV promoter to produce an intact anti-

18 body,

19 transcription of a single RNA comprising protein sub-units and

20 selection marker.

21

22 Employing the method according to the invention it has proven

23 that the kappa light chain and gamma heavy chains are expressed

24 in about equimolar quantity. The antibody concentration obtained

25 proved to be at least 1µg/ml, preferably 5-300 µg/ml.

26

27 The following examples are describing the invention in more de-

28 tail, but not limiting the scope of the invention.

29

30 E x a m p l e s

31

32 I. Production of recombinant mouse IgG2a mAb17-1A antibody (r

33 mAb17-1A,)

34

35 **Example 1: Molecular biological constructs**

36

37 The bicistronic pIRES expression vector (Figure 1) purchased

38 from Clontech laboratories Inc., Palo Alto, USA allows to ex-

39 press two genes at high level and enables the translation of two

40 consecutive open reading frames from the same messenger RNA. In

1   order to select positive transformants using a reporter protein,
2   the internal ribosome entry site (IRES) in this expression vec-
3   tor has been truncated enabling lower expression rates of this
4   second reading frame. Therefore, the original IRES sequence had
5   to be re-established in order to satisfy our purposes expressing
6   heavy and light antibody chain at nearly the same expression
7   level. The attenuated IRES sequence is used for the expression
8   of our selection marker.
9
10   DNA manipulations were done by standard procedures. Using PCR
11   technology and the Advantage-HF PCR Kit (CLONTECH laboratories
12   Inc., Palo Alto, USA), the heavy and the light chain of the
13   mAb17-1A (HE-2) antibody were amplified using primers introduc-
14   ing the respective cleavage sites for restriction endonucleases
15   necessary for the introduction of the gene into the expression
16   vectors once and twice the Kozak-sequences upstream of the open
17   reading frames. The autologous signal sequences were used to di-
18   rect nascent polypeptide chains into the secretory pathway.
19   Primers were purchased from MWG-Biotech AG, Germany. Figure 2
20   shows the cloning cassette used for the bicistronic expression
21   of mAb17-1A (HE-2). A two step cloning strategy was performed:
22   Kappa-chain including its autologous signal sequence was ampli-
23   fied as Xho I, Mlu I fragment and ligated into the expression
24   vector using the Rapid ligation kit (Roche, Germany) according
25   to the instructions of the manufacturer. The construct was
26   transfected into chemical competent E. coli bacterial strain
27   DH5alpha, (Gibco BRL) and amplified using the ampicilline selec-
28   tion marker. In a second step, the reconstructed IRES sequence
29   and Gamma chain, also including its autologous signal sequence,
30   were amplified as Mlu I, Nco I and Nco I, Sal I fragments re-
31   spectively and ligated in a single step ligation reaction into
32   the modified expression vector already containing the mAb17-1A
33   Kappa chain. This construct was amplified using the bacterial
34   strain DH5alpha (Gibco BRL). Twenty-five constructs deriving
35   from different PCR samples were digested using the restriction
36   endonucleases EcoR I and BamH I. Constructs showing the correct
37   digestion map were bi-directionally sequenced. In this expres-
38   sion construct, the selection cassette described below was in-
39   troduced. The selection marker DHFR was amplified as PCR Xba I /
40   Not I fragment from the pSV2-dhfr plasmid (ATCC #37146). PCR-

1   primers introduced these restriction sites. The attenuated IRES
2   at. sequence was amplified by PCR from pSV-IRES (Clontech #6028-
3   1) as Sal I / Xba I fragment. In a single step ligation reac-
4   tion, IRES at. and DHFR was ligated into the already described
5   expression construct after being digested with the corresponding
6   restriction endonucleases and a further dephosphorylation step.
7   After a transfection into the bacterial strain DH5alpha (Gibco
8   BRL), positive transformants were screened by PCR. The correct
9   insertion of selection and expression cassettes was proven by
10 minipreparation and further digestion-map shown in Figure 2.
11 The constructs were bi-directional sequenced and used in further
12 transfections in eukaryotic cells.
13
14 **Example 2: Transfection**
15
16 The characterized eukaryotic strain, CHO (ATCC-CRL9096), was
17 transfected with the expression vector prepared as described
18 above. The DHFR selection marker was used to establish stable
19 cell lines expressing rmAb17-1A. In a six-well tissue culture
20 plate, the cell line was seeded at densities of 105 cells in 2
21 ml complete Iscove's modified Dulbecco's medium with 4 mM L-
22 glutamine adjusted to contain 1.5 g/L sodium bicarbonate and
23 supplemented with 0.1 mM hypoxanthine and 0.016 mM thymidine,
24 90%; fetal bovine serum, 10% (Gibco.BRL). Cells were grown until
25 50% confluency. Cells were transfected according to the instruc-
26 tions of the manufacturer in absence of serum with 2 μg DNA us-
27 ing Lipofectin☐ reagent (Gibco-BRL). Transfection was stopped by
28 addition of complete medium after 6 or 24 hours.
29
30 **Example 3: Selection of positive transformants and cultivation**
31
32 Complete medium was replaced by selective medium 24 or 48 hours
33 post transfection. FCS in complete medium was replaced by dia-
34 lyzed FCS (Gibco.BRL, origin: south America). 10 days post se-
35 lection, positive transformands appeared as fast growing multi-
36 cellular conglomerates. Concentration of rmAb17-1A was analyzed
37 in supernatants by a specific sandwich ELISA recognizing both
38 the variable and the constant domain of the antibody. Cells
39 showing high productivity were splitted 1:10 and expanded into
40 75 cm2 cell culture flasks for preservation into liquid nitro-

1 gen. In parallel, these producers were exposed to an increasing
2 selection pressure by adding Methotrexate to the culture medium
3 and seeding the cells into a six-well cell culture plate. Proce-
4 dure was repeated about two weeks later when cells reached sta-
5 ble growth kinetics. Starting from a concentration of 0.005 μM,
6 MTX concentration was doubled each round of selection until fi-
7 nally a concentration of 1.280 μM MTX was reached and sub cul-
8 tured in parallel into 96-well tissue culture plates. Super-
9 natants were analyzed weekly by a specific sandwich ELISA recog-
10 nizing both the variable and the constant domain of the anti-
11 body. Stable cultures showing highest productivity were trans-
12 ferred into 75-cm2 cell culture flasks and stepwise expanded fi-
13 nally into 860-cm2 rolling tissue culture flasks in non selec-
14 tive medium. Supernatants were harvested, centrifuged, analyzed
15 and submitted to further purification.
16
17 **Example 4:**
18
19 Production of rmAb17-1A under serum free conditions.
20 Recombinant rmAb17-1A was produced in lab-scale by engineered
21 CHO cell-line using protein free medium EXCELL® 325PF (JRH Bio-
22 sciences) in roller-bottles. The supernatants were affinity pu-
23 rified using the anti-idiotypic antibody IGN111 immobilized onto
24 SEPHAROSE® and characterized by SDS-PAGE, SEC-HPLC, ELISA and
25 IEF.
26
27 **Example 5: Analysis of expression products**
28
29 Supernatants were analyzed by specific ELISA recognizing both,
30 the variable and the constant domain of the expressed antibody.
31 The polyclonal anti-idiotypic antibody IGN111 was coated at 10
32 μg/ml onto MAXISORP™ (NUNC) sorption plates. This anti-idiotypic
33 antibody was raised by immunization with mAb17-1A F(ab)2 frag-
34 ments. The induced overall immune response was negatively affin-
35 ity purified using immobilized 16B13ab, a murine IgG2a antibody
36 of identical isotype but different specificity. Flow through
37 fractions were affinity purified using immobilized mAb17-1A
38 F(ab)2. Remaining antibodies against mouse constant regions were
39 absorbed to a column on which polyclonal mouse IgG was immobi-
40 lized. The final product, the polyclonal IGN111 antibody prepa-

1 ration thus recognizes the variable domain of mAb17-1A. Remain-
2 ing active groups were blocked by incubation with 1% skim milk
3 and supernatants were applied. Expressed antibodies were de-
4 tected by their constant domains using a rabbit-anti-mouse-
5 IgG2a-HRP conjugate (Biozym). Quantification was performed by
6 comparison to an also loaded and characterized mAb17-1A standard
7 hybridoma antibody.

8

9 Size determination of expressed proteins was performed by SDS-
10 Polyacrylamide gel electrophoresis using 4-14 % acryl amide gra-
11 dient gels in a NOVEX™ (Gibco-BRL) electrophoresis chamber. Pro-
12 teins were silver-stained. To detect the expressed antibodies
13 immunologically, Western-blots were carried out on nitro-
14 cellulose membranes (0.2 μm). Proteins separated on SDS-
15 Polyacrylamide gels were electro transferred using a NOVEX™
16 (Gibco-BRL) blotting-chamber. The membranes were washed twice
17 before adding blocking solution (TBS + 3 % Skim Milk Powder BBL)
18 and the antibody solution (10 μg/ml polyclonal goat IGN-111 an-
19 tibody, mouse monoclonal anti-mouse IgG antibody (Zymed) or rab-
20 bit anti-mouse IgG gamma chain (Zymed) in TBS + 1 % Skim Milk
21 Powder). Finally development was performed using a rabbit anti-
22 goat-HRP, rabbit anti-mouse IgG-HRP or mouse anti-rabbit IgG-HRP
23 conjugated antibody (BIO-RAD) diluted at 1:1000 in TBS + 1 %
24 Skim Milk Powder and an HRP color development reagent (BIO-RAD)
25 according to the manufacturers instructions.

26

27 Isoelectric focusing gels were used to compare the purified ex-
28 pression products to the characterized murine mAb17-1A standard
29 hybridoma antibody. Samples were loaded onto IEF gels, pH 3-7
30 (Invitrogen) and separation was performed according to the in-
31 structions of the manufacturer. Proteins were visualized by sil-
32 ver stain or by immunological methods by Western-blot. For this
33 purpose, proteins were charged in a Tris buffered
34 SDS/Urea/Iodoactamide buffer and transferred onto nitro-
35 cellulose membranes using the same procedure described for West-
36 ern-blots. Detection was performed using the polyclonal goat
37 IGN111 anti-idiotypic antibody.

38

39 Interaction of expression products with their target antigen,
40 EpCAM was analyzed by incubating purified supernatants with Ni-

1 tro-cellulose membranes on which rEpCAM was electro-transferred.
2 Staining of interacting antibodies was performed in analogy to
3 Westen-blots using an anti-mouse IgG2a-HRP conjugated antibody
4 (Zymed).
5
6 **Example 6: Affinity purification**
7
8 A Pharmacia (Amersham Pharmacia Biotech) ÄKTA system has been
9 used. 1000 ml clarified culture supernatant containing antibody
10 were concentrated using a Pro-Varion 30 kDa cut-off (Millipore)
11 concentrator, then diluted with PBS and loaded onto a 20 ml
12 IGN111 SEPHAROSE® affinity gel XK26/20 column (Amersham Phar-
13 macia Biotech). Contaminating proteins were discarded by a wash
14 step with PBS + 200 mM NaCl. Bound antibodies were eluted with
15 100 mM Glycine, pH 2.9 and neutralized immediately using 0.5 M
16 NaHCO3. Effluent was online monitored at Λ 215 and Λ 280 nm and
17 submitted to a subsequent HPLC analysis using a ZORBAX® G-250
18 (Agilent-technologies) column.
19
20 2000 ml harvested supernatants, deriving from roller bottle cul-
21 tures were centrifuged, concentrated, diluted in PBS and puri-
22 fied to homogeneity by affinity chromatography using the IGN111
23 SEPHAROSE® column. After elution, neutralization and dialysis
24 against PBS, final product was measured by SEC-HPLC. A hybridoma
25 derived murine standard of the same immunoglobulin was compared
26 with rmAb17-1A and eluted, both as sharp single peaks, at the
27 same time, correlating with the expected retention time of IgG.
28 Purity >92 % was reached using this laboratory scale purifica-
29 tion strategy.
30
31 Further characterization of the expression product was carried
32 out by reducing and non reducing silver stained SDS-PAGE and
33 Western-Blot. The expression products were detected by the spe-
34 cific, anti-idiotypic antibody goat anti mAb17-1A, IGN111, and
35 visualized by an anti-goat-HRP conjugated antibody. Not reduced
36 samples showed bands in the expected range of an intact IgG
37 molecule corresponding to 160 kDa. This result correlates ex-
38 actly with the murine standard mAb17-1A hybridoma antibody. In
39 the case of reduced samples, bands in the range of 25 and 50
40 kDa, also interacting with the anti-idiotypic goat anti mAb17-1A

1 antibody IGN111, are visible. Those bands correspond to IgG
2 light and heavy chains respectively.
3
4 Interaction with the target antigen of mAb17-1A, EpCAM was ana-
5 lyzed by incubating Nitro-cellulose membranes on which rEpCAM
6 has been electro-blotted, with purified expression products.
7 Further subtype specific detection of interacting antibodies was
8 done. The murine mAb17-1A standard hybridoma antibody recognizes
9 the monomeric rEpCAM of 25 kDa and also a series of rEpCAM ag-
10 gregates, corresponding to di, tri, and polymeric forms. Exactly
11 the same band distribution is found for all purified expression
12 products.
13
14 Purified expression products and the murine mAb17-1A standard
15 hybridoma antibody were further analyzed. All antibodies show an
16 inhomogeneous polybanded isoelectric focusing-pattern, identical
17 in pH but different in quantitative distribution, consisting in
18 three major protein isoforms and two sub forms, distributed over
19 a pH range of 8.2 to 7.2. CHO derived isoforms are shifted to
20 higher pH values, the murine mAb17-1A standard shows the identi-
21 cal isoforms, but quantitative distribution tends towards acidic
22 forms.
23
24 We were able to express recombinant mouse IgG2a antibody mAb
25 17-1A in CHO cells. Stable genomic integration occurred 14 days
26 after transfection. The expression construct enabled rapid and
27 comfortable transfection using a single plasmid. By the use of a
28 selection system based on an essential metabolic enzyme depleted
29 host strain, a plasmid carrying the corresponding gene ant a po-
30 tent antagonist of this enzyme, gene copy number could be in-
31 creased by continuous increasing selection pressure. The use of
32 an attenuated IRES sequence in the expression cassette of this
33 selectable marker, very low amounts of the antagonist MTX could
34 be used for the selection strategy. Moderate expression was
35 achieved with levels about $10 \mu g$ /24 h.ml, which could be kept at
36 least 5 weeks in production cultures. Purified expression prod-
37 ucts did not differ from the murine mAb 17-1A standard in size
38 and specific immunological essays. Nevertheless, differences in
39 post translatorial modifications may have occurred. Therefore,
40 recombinant antibodies showed a host or medium specific isoelec-

1 tric focusing pattern. Biological equivalence of the expression
2 product are further analyzed in immunization studies.
3
4 **Example 7: Rhesus Monkey Immunization Study**
5
6 Study Protocol
7
8 A Rhesus monkey immunization study was performed at BioTest
9 s.r.o. facilities (Conarovice, CZ). Immunogenicity of IGN101
10 (mAb17-1A) and IGN101 (recombinant-mAb17-1A) was compared in na-
11 ïve Rhesus monkeys. Each treatment group consisted of 2 male and
12 2 female monkeys (4-6 kg body weight). A single dose of 0.5 mg
13 of the respective mAb17-1A formulated onto Al(OH)3 was adminis-
14 tered subcutaneously on days 1, 15, 29 and 57. Serum samples
15 were taken from monkeys 11 days before first vaccination and on
16 study days 1, 15, 29, 57, and 71. Serum samples were taken be-
17 fore each vaccination. All serum samples taken before immuniza-
18 tion (i.e. day -11 and day 1) are considered as pre-immune sera
19 (Pre-IS).
20 Immunogenicity was assessed as a primary objective of this
21 study:
22 • Humoral immune response to the mAb17-1A antigen was examined by
23   ELISA and by immunization antigen specific affinity chromatog-
24   raphy.
25 **Preparation of Study Medication**
26 As mentioned above 2 types of drug substance (mAb17-1A) were
27 used this study: hybridoma-derived mAb17-1A and recombinant
28 mAb17-1A (lab scale). All types were adsorbed onto Al(OH)3 in
29 the same amounts and concentrations.
30 Recombinant mAb17-1A
31 r-mAb17-1A was produced in lab-scale by the engineered CHO
32 cell-line (E5 WCB 325 R11/1a) in roller-bottles using protein-
33 free medium EXCELL® 325 PF (JRH Biosciences). The supernatant
34 was affinity purified using Protein A SEPHAROSE®. Purified re-
35 combinant mAb17-1A was characterized by SDS-PAGE, SEC-HPLC,
36 ELISA and IEF.
37 **Analysis of Immune Response**
38 Immunization antigen-specific (mAb17-1A) ELISA
39 Method description
40 Pre-immune sera and immune sera of different time points were

1 analyzed by an immunization antigen-specific ELISA recognizing

2 induced humoral immune response. This was performed using mAb17-

3 1A as coating antibody coated at 10 μg/ml onto MAXISORP™ (NUNC)

4 sorption plates diluted in coating buffer (PAA). Remaining ac-

5 tive groups were blocked by incubation with 3% FCS (Gibco BRL,

6 heat inactivated) in PBS before sera were applied in 6 x 1:3 di-

7 lutions in PBS supplemented with 2% FCS. Induced antibodies were

8 detected by their constant domains using a rabbit-anti-human-

9 IgG, A, M-HRP conjugate (Zymed). Staining was performed by OPD

10 (Sigma) in staining buffer (PAA) using H2O2 as substrate accord-

11 ing to the manufacturer's instructions. Absorbance at 492 nm was

12 measured using 620 nm as reference wavelength. Quantification

13 was performed by comparison with a loaded and characterized

14 Rhesus monkey immune serum of a previous immunization study

15 (8415F day 94), which is standardized equivalent to a titer of

16 1:9000.

17 <u>Results and discussion</u>

18 Substantial titers of antibodies against mAb17-1A were induced

19 in all 2 treatment groups: Antibody titers against mAb17-1A ap-

20 peared on day 15, remaining at a high level between day 29 and

21 day 71 (Table 1). There was no significant difference in kinet-

22 ics and extent of the immune response induced either by IGN101

23 (mAb17-1A) or IGN101 (r-mAb17-1A).

24

25

Table 1: Immunization antigen (mAb17-1A)-specific titer (ELISA)

Treatment group animal number	Day of treatment: 0	8	15	29	57	71
mAb17-1A						
128	1*	1	653	1561	1844	7940
150	1	1	1300	30693	16976	20106
109	1	1	8040	33000	27160	49885
289	1	1	11255	23435	18863	36197
Geometric mean	1	1	2960	13874	11253	23171
CI+	1	1	20204	105838	61407	71032

Cl-	*1*	*1*	*434*	*1819*	*2062*	*7559*
r-mAb17-1A						
140	1	1	1156	6296	4151	15072
265	1	1	8948	18189	19776	45544
184	1	1	8221	24846	5672	26012
121	1	1	37	369	3894	23367
Geometric mean	**1**	**1**	**1332**	**5692**	**6525**	**25415**
Cl+	*1*	*1*	*47115*	*81371*	*18666*	*47789*
Cl-	*1*	*1*	*38*	*398*	*2281*	*13516*

* values below detection limit were replaced by '1' for statistical evaluations

1

2

3

4 Affinity chromatography

5 <u>Rationale and method description</u>

6

7 The amount of IgG and IgM of total antibodies induced against

8 the respective immunization antigen (mAb17-1A or r-mAb17-1A)

9 were quantified as follows: In a first step the respective immu-

10 nization antigen was coupled to CH-SEPHAROSE® 4B (2 mg/ml) and

11 filled into a 1 ml chromatography column. 1.0 ml of monkey serum

12 (pre-immune (day -11) and immune sera from day 29, 57 and 71)

13 was diluted 1:10 in running buffer (PBS supplemented with 200 mM

14 NaCl) and loaded onto the column. The unbound sample was washed

15 out with running buffer. Fractions of interest containing the

16 antigen-specific humoral immune response were desorbed with elu-

17 tion buffer (100 mM Glycine/HCl, pH=2.9) and collected by auto-

18 mated fractionation and immediately neutralized by adding 1.0 M

19 NaHCO3.

20

21 Total immunoglobulin concentration and IgG and IgM ratio in

22 eluted fractions were determined by size exclusion chromatogra-

23 phy using a ZORBAX® GF 250 column. Commercially available, poly-

24 clonal human IgG and IgM (PENTAGLOBIN®) was used as standard.

25

1 Results and discussion

2 Induced immunization antigen specificity

3

4 All two treatment groups raised a strong immunization antigen-
5 specific IgG immune response (Table 2). IgG increased in all
6 groups from day 29 to 71. Levels of immunization antigen-
7 specific immune titres were found to be very similar in groups
8 vaccinated with either IGN101 (mAb17-1A) or IGN101 (r-mAb17-1A).
9 Due to small group size and interindividual variability no sig-
10 nificant differences could be determined.

11

Table 2: Induced immunization antigen-specific IgG (µg IgG/ml; affinity chromatography)

Treatment group/ animal number	Day of treatmen			
	-11	29	57	71
mAb17-1A				
128	13,2	15,4	59	126,9
150	b.d.	128,4	232,6	257,4
109	b.d.	232,2	203,9	436,5
289	b.d.	97,6	122,1	184,4
Average	**3,3***	**118,4**	**154,4**	**251,3**
standard deviation	*6,6*	*89,6*	*79,0*	*134,5*
CI	*9,2*	*124,4*	*109,6*	*186,7*
r-mAb17-1A				
140	b.d.	20	102,11	202,105
265	b.d.	116,7	104,73	217,4
184	b.d.	93,8	225,88	283,6
121	b.d.	55,2	97,12	243,7
Average		**71,4**	**132,5**	**236,7**
standard deviation		*42,7*	*62,4*	*35,7*
CI		*59,2*	*86,6*	*49,5*

n.a. not analyzed

b.d. below detection limit (i.e. 12.0 µg/ml)

* for statistic calculations values below detection limit were set '0'

1

2

Table 3: Induced immunization antigen-specific IgM (µg IgM/ml; affinity chromatography)

Treatment group/ animal number	Day of treatment: -11	29	57	71
mAb17-1A				
128	31,8	34,8	19,6	28,9
150	b.d.	19,5	22	20,1
109	b.d.	16,7	20,3	24
289	b.d.	13,1	13,8	14,3
Average	**8***	**21,0**	**18,9**	**21,8**
standard deviation	*15,9*	*9,5*	*3,6*	*6,2*
CI	*22,1*	*13,3*	*4,9*	*8,6*
r-mAb17-1A				
140	b.d.	6,9	9,5	19,65
265	6,8	9,3	19,4	23,9
184	6,3	7,1	18,6	22,15
121	30,1	73,5	40,8	37,38
Average	**14,4**	**24,2**	**22,1**	**25,8**
standard deviation	*13,2*	*32,9*	*13,3*	*7,9*
CI	*18,4*	*45,6*	*18,4*	*11,0*

n.a. not analyzed

b.d. below detection limit (i.e. 3.5 µg/ml)

* for statistic calculations values below detection limit were set '0'

1 'Cross comparative' ELISA

2 <u>Rationale and method description</u>

3 This assay was carried out with immune-sera (day 71) of Rhesus

4 monkeys vaccinated with either IGN101 (mAb17-1A) or IGN101 (r-

5 mAb17-1A). The aim of the 'cross comparative ELISA' is to di-

6 rectly compare e.g. epitope specificity of the respective immune

7 responses of the two vaccine antigens:

8 1)Antibodies induced by IGN101 (mAb17-1A) immunization are ap-

9 plied to ELISA plates coated with mAb17-1A or r-mAb17-1A.

10 2)Binding activity of antibodies induced by IGN101 (rmA17-1A)

11 immunization are tested on ELISA plates coated with mAb17-1A or

12 r-mAb17-1A.

13 <u>Results and discussion</u>

14 Figure 10 shows the results of the experiment. Cross-

15 comparative ELISA analysis. Geometric means (4 animals per

16 group) and CI (95%) are shown.

17 No difference in humoral immune response was found comparing im-

18 mune sera induced by vaccination with either IGN101 (mAb17-1A)

19 or IGN101 (r-mAb17-1A) regarding mAb17-1A or r-mAb17-1A binding

20 specificity. Single values of each Rhesus monkey are given in

21 Annex 1. Results suggest that exactly the same immunogenic epi-

22 topes are presented in both types of vaccines.

23

24 **Repeated Dose Safety Pharmacology and Toxicity Study**

25 A 13-week safety pharmacology study has started in November 2003

26 at Covance Laboratories GmbH (Münster, Germany). This study is

27 conducted in compliance with the Good Laboratory Practice Regu-

28 lations. As for previous animal studies, Rhesus monkeys (Macacca

29 mulatta) are used for toxicity testing.

30

31 Dose, vaccination schedule, and administration of the test sub-

32 stance reflect the intended clinical use as well as previous

33 animal studies and numerous clinical trials performed with

34 IGN101 (mAb17-1A):

35

36 Primary vaccination are being performed on days 1, 15, and 29.

37 On day 57 a booster injection is given. All injections are ad-

38 ministered subcutaneously in a volume of 0.5 ml per single dose.

39 As in a previous study, the total observation period was set to

40 93 days. Dose selection is based on considerations outlined in

1   the description of the previous animal study: 500 μg mAb17-1A

2   (~90 μg/kg), adsorbed on aluminum hydroxide per single dose.

3   One treatment group is immunized with IGN101 (mAb17-1A), a sec-

4   ond receives IGN101 (r-mAb17-1A). The recombinant antibody stems

5   from a GMP batch. The placebo group is treated with the equiva-

6   lent formulation lacking the antibody compound.

7   Each treatment group consists of 2 male and 2 female Rhesus mon-

8   keys (n=4).

9

10   Clinical and physiological examinations are being performed in

11   all animals. Food intake, general behavior and body weight are

12   recorded at regular intervals. Haematological, immunological pa-

13   rameter, urinalysis and parameter of clinical chemistry are de-

14   termined at relevant intervals (bleeding schedule, outlined be-

15   low).

16

17   **Terminal Monitoring**

18   Autopsy will be conducted on all animals. Organ weights, macro-

19   scopic and histopathological observations are recorded for all

20   commonly examined tissues. Tissue samples are conserved for fur-

21   ther examinations.

22

23   **Pharmacodynamics**

24   Immunological analyses are included into repeated dose toxicity

25   and take into account the pharmacodynamic and -kinetic profiles

26   as obtained from the previous animal study, clinical trials and

27   results published from related studies (Galili, U. (1993) Inter-

28   action of the natural anti-Gal antibody with alpha-galactosyl

29   epitopes: a major obstacle for xenotransplantation in humans.

30   Immunology Today; 14(10): 480-2, Frodin, J. E., Lefvert, A. K. &

31   Mellstedt, H. (1990). Pharmacokinetics of the mouse monoclonal

32   antibody 17-1A in cancer patients receiving various treatment

33   schedules. Cancer Res 50, 4866-71.). Specific ELISAs as well as

34   chromatographic approaches are performed to quantify and charac-

35   terize the immunological response in blood samples:

36   a)   Total immune response is shown by an ELISA specific for the

37   immunization antigen (mAb17-1A). A subclass ELISA is performed

38   to characterize the type of immune response. A 'cross compara-

39   tive ELISA' is performed to examine immune sera from animals

40   vaccinated with recombinant mAb17-1A by comparing their binding

1   properties to the immunization antigen (i.e. r-mAb17-1A) as
2   well as to the hybridoma-derived mAb17-1A. This is done vice
3   versa with sera of animals vaccinated with the hybridoma
4   mAb17-1A. It is anticipated that the immune sera display simi-
5   lar binding properties irrespective of the antibody coated to
6   the ELISA plates.
7   b)   Target antigen-specific antibody reactions will be demon-
8   strated with a sequential affinity chromatography.
9
10   In addition to final observations these parameters are monitored
11   with a frequency that permits an assessment of changes over
12   time: Blood samples for immunological analysis and kinetics are
13   taken once before the start of study (day -14), on day 1 (di-
14   rectly prior to vaccination, 1, 4 and 24 hours after vaccina-
15   tion) and on days 43, 71 and 92 in the morning and at necropsy
16   during exsanguination (day 93).
17
18   Specific studies for Al(OH)3 are not being performed, since the
19   profile of the commonly used adjuvant has been examined and well
20   documented (Weiner, L. M. et al. (1993). Phase II multicenter
21   evaluation of prolonged murine monoclonal antibody 17-1A therapy
22   in pancreatic carcinoma. J Immunother 13, 110-6)
23
24   The metabolic pathway of antibodies is well understood, thus ob-
25   viating the need of biotransformation studies.
26
27   **Local Tolerance**
28   Testing for local tolerance is included within repeated dose
29   toxicity study.
30
31   **Preliminary Results**
32   The first of four subcutaneous vaccinations of IGN101 was well
33   tolerated and did not reveal any adverse toxic signs: There were
34   no clinical signs that could be ascribed to treatment with the
35   test article. No skin changes at the injection sites were ob-
36   served and no signs of abnormal local tolerance were reported.
37
38   **Summary and Conclusion**
39   First results of serum sample analyses of monkeys vaccinated
40   with either IGN101 (mAb17-1A) and IGN101 (r-mAb17-1A) show that

1 both types of antigens induce a comparable immune response in
2 Rhesus monkeys. Moreover, the extent of induced immune response
3 was found to be essentially similar in both groups.
4
5 Side-by-side biochemical characterization of both vaccine anti-
6 gens has shown that the two antigens are very similar in protein
7 structure and binding activity. In addition, it was shown that
8 the immune response elicited by both vaccine antigens was found
9 to be essentially similar in quality and quantity as analyzed so
10 far. Igeneon will pursue the characterization of the immune re-
11 sponse induced in Rhesus monkeys but also in patients to verify
12 the hypothesis that the immune response induced by either vac-
13 cine antigen will be essentially similar.
14

Table 4: Induced immunization antigen-specific titres ('Cross comparative' ELISA)

Treatment group/ animal number	Coated with r-mAb17-1A	Coated with mAb17-1A
mAb17-1A		
128	6520	8326
150	25371	24733
109	21559	22682
289	13486	19621
geomean	**14809**	**17399**
CI+	*34485*	*34855*
CI-	*6359*	*8685*
r-mAb17-1A		
140	12789	12822
265	12946	12237
184	22009	20350
121	16172	16489
geomean	**15581**	**15148**
CI+	*22176*	*21031*
CI-	*10947*	*10910*

15
16

1

2 Results

3 Considering all vaccinations, no side effects were observed.

4 In this immunization study, the vaccination with different IgG2a

5 formulations induced in all cases a strong IgG type immunization

6 antigen specific immune response. Except for the deglycosylated

7 17-1A formulation which caused a lower immune response, the im-

8 munogenicity of all other formulations was nearly the same. Im-

9 mune titers increased from values below the detection limit up

10 to 300 μg/ml serum corresponding to an induced IgG ratio of

11 nearly 1%. Immunogenicity of all applied glycosylated IgG2a an-

12 tibodies was nearly in the same range, independent from their

13 specificity.

14

15 Also independent from the immunization group, all IgG2a vacci-

16 nated animals raised an IgG type immune reponse recognizing Ep-

17 CAM corresponding to an amount of 30-40% of the immunization an-

18 tigen specific titer. Vaccination with IgG2a antibodies caused

19 therefore a cross reactivity of the immune sera with EpCAM. De-

20 glycosylation of the immunization antigen decreased both induced

21 IgG levels significantly, the ones directed against the immuni-

22 zation antigen and the ones against EpCAM.

23

24 Deglycosylation considerably changes the immunogenetic proper-

25 ties of the antibody. Both the immunoglobulin titers against the

26 immunization antigen and the target antigen were reduced.

27

28 The comparison between the original, hybridoma derived immuniza-

29 tion antigen 17-1A and the recombinantly expressed r mAb 17-1A

30 from CHO cells did not reveal any immunological differences.

31 Both formulations showed identical kinetics building up the im-

32 munization antigen and target antigen specific immune response.

33 Raised IgG and IgM titers were similar.

34

35 **Example 8: Expression of a hybrid immunogenic antibody**

36

37 The recombinant IgG2a Le-Y antibody is an IgG2a hybrid antibody

38 designed for primate vaccination. It combines an anti-idiotypic

39 Lewis-Y (Le-Y) mimicking hypervariable region and the highly im-

40 munogenic mouse IgG2a constant regions.

1

2   A figure of the IgG2a Le-Y antibody is shown in Fig 4.

3   The recombinant IgG2a Le-Y antibody immunotherapy enhances the

4   immunogenicity of the parent antibody IGN301 produced by a hy-

5   bridoma cell. It induces a strong IgG type immune response di-

6   rected against Le-Y and / or EpCAM overexpressed and presented

7   on epithelial cancer cells. This immune response lyses tumor

8   cells by complement activation or cell mediation preventing the

9   formation of metastases.

10

11 Molecular biological constructs of the recombinant IgG2a Le-Y

12 antibody were incorporated into the poly-cistronic expression

13 vector described above as shown in Figures 1 and 2.

14

15 The recombinant IgG2a Le-Y antibody was expressed transiently

16 in HEK293 cells calcium phosphate co-precipitation in a Micro-

17 Spin system in presence of FCS. After purification using an

18 anti-Le-Y affinity column and qualification of the expression

19 product, the recombinant IgG2a Le-Y antibody was formulated

20 onto Al(OH)3 and administrated as vaccine in a Rhesus monkey im-

21 munization study using four 500 μg doses.

22

23 High immunogenicity in comparison with the parent vaccine IGN301

24 could be observed. The induced IgG type immune response was ana-

25 lysed by ELISA and showed an immunisation antigen, Le-Y speci-

26 ficity.

27